Anti-Nucleoprotein Antibody Response in Influenza A Infection

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SUKENO, N., OTSUKI, Y., KONNO, J., YAMANE, N., ODAGIRI, T., ARIKAWA, J. and ISHIDA, N. *Anti-Nucleoprotein Antibody Response in Influenza A Infection*. Tohoku J. exp. Med., 1979, 128 (3), 241-249 — The nucleoprotein (NP) antigen isolated from influenza A virus by solubilization with Triton X-100 (TX-100) and further electrophoresis with SDS-cellulose acetate membrane gave a single band on SDS-polyacrylamide gel electrophoresis. Rabbit anti-serum hyperimmunized with the NP reacted only against the NP antigen. Moreover, a well-defined single precipitin line was shown between the NP and human sera. These results suggested that the NP was possible to detect anti-NP antibody in human serum. Immuno double diffusion (IDD) and single radial immunodiffusion (SRD) tests using the NP were established to detect the anti-NP antibody in human sera. During an epidemic caused by antigenic drift strain, anti-NP antibody was detected by the IDD test in the cases which did not show any significant rise in HAI titer. During a mixed epidemic caused by the different strain of HA antigen, the infection ratio in mass population was revealed more convenient and sensitive by SRD than HAI. The anti-NP antibody was detected by IDD for long periods of one year or more after infection. These results suggest that the detection of anti-NP antibody is applicable to serologic studies, particularly serologic diagnosis and serologic surveys of influenza infection in mass population. — influenza A; anti-nucleoprotein antibody; immunoprecipitation test; sero-diagnosis

The standard hemagglutination-inhibition (HAI) test based on the principles described by Hirst and Salk (Hirst 1941; Salk 1944) is currently used as the routine assay system in the serodiagnosis and in carrying out the sero-epidemiology of influenza virus. Although the HAI test is largely specific to the antibody of each hemagglutinin and believed to reflect the neutralizing activity, there still exists a number of variable factors, i.e. the presence of non-specific inhibitors (alpha, beta and gamma) and the variability among the test strains in their avidity or affinity to chicken erythrocytes (Kilbourne 1975). The most troublesome factor in making the serodiagnosis is the selection of the test strain, when immediate information is requested. It is well known that the antigenic determinants of hemagglutinin of influenza A virus frequently change, i.e. antigenic drift and

shift (Webster and Laver 1971). In addition, although the most recent isolate provides the best antigen for the HAI test in determining influenza infection, under the present conditions the viral isolation usually lags behind the clinical outbreak of the influenza pandemic.

On the other hand, the internal proteins of influenza virus (nucleoprotein and membrane protein) have type-specific antigens which are believed to be identical among the various strains of each type of influenza virus (A, B, C) (Schild and Pereira 1969; Schild 1972; Kilbourne 1975; Schild et al. 1975). Therefore, serodiagnosis using the internal proteins should be easier as there is no effect from antigenic drift or antigenic shift. Initially, the complement fixation (CF) test was the only available method for the assay of the antibody to the internal antigens (Lief and Henle 1959). The CF test was routinely performed using the so-called "soluble antigen" extracted from infected chorioallantoic membranes. However, this test is not so useful because its sensitivity and specificity are undoubtedly low and the CF titers immediately disappear after natural infection (Stuart-Harris and Schild 1976).

Therefore, we established another assay system based on the immuno double diffusion (IDD) and single radial immunodiffusion (SRD) tests to detect the antibodies to internal proteins particularly the nucleoprotein.

This publication describes, first, the isolation of the nucleoprotein from the influenza A virion in an immunologically and physico-chemically pure form, and second, how to utilize the isolated nucleoprotein to make the serodiagnosis of influenza infection, and also includes a comparative study using the HAI test on the actual pandemics of 1976-78.

MATERIALS AND METHODS

Virus strains. The Hong Kong strain of type A influenza virus A/Miyagi/1/76 (H3N2) isolated in Miyagi prefecture was used as the starting material for the NP antigen. A/Victoria/3/75 (H3N2), A/Texas/1/77 (H3N2) and A/USSR/90/77 (H1N1) were used as the antigens in the HAI tests.

Immunodiffusion agar. Agarose (Indubiose A-37, Industrie Biologique Francaise S.A. Gennevilliers, France) was used in the immunoprecipitin test. The agar gel was prepared at a final concentration of 0.9% agarose in 0.01M tris-HCl-saline buffer (TSB), pH 7.5, and contained 0.1% TX-100.

Preparation of immunoplates for immuno double diffusion (IDD) test. A 5.5 ml volume of A-37 agarose melted by heating was added to each slide-glass (2.5 by 7.5 cm) on a horizontal surface. After solidifying, six circular wells surrounding one central well were cut in the gel using a 3 mm diameter punch and a template. Eight μ l of NP antigen were added to the central well and six surrounding wells accommodated the anti-NP antibodies.

Preparation of immunoplates for single radial immunodiffusion (SRD) test. Agarose gel was melted in a boiling water bath and cooled to 56°C before the addition of NP antigen. The agar and appropriate amount of antigen were mixed by vigorous shaking and 1.5 mm layers of gel were made in round plastic immunoplates (Kowa Laboratory, Nagoya) using 2.5 ml of agarose per plate. Circular wells (3.0 mm diameter) were cut in the agarose to accommodate the detection of the anti-NP antibodies. Eight microliter volumes of sera were added to the wells and the plates incubated in a moist chamber at 37°C for 16 hr. The presence of antibodies to NP antigen in the sera was detected by the appearance of

opalescent zones surrounding the wells. Accurate measurements of the zone diameters were made under darkground illumination using a micrometer scale.

Hemagglutination inhibition test (HAI). HAI tests were performed in microtiter V plates using receptor-destroying-enzyme treated sera, according to the method of the WHO (Palmer et al. 1975). 0.5% chick red blood cell suspension and 4 HA units of virus infected chorioallantoic fluid were used in the HAI test.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was performed in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) according to the method of Davis (1964). After electrophoresis, the protein was fixed and stained by 1.0% Coomassie brilliant blue dye in 7% acetic acid. The positions of the polypeptide bands were determined by scanning the gels at 580 nm using a densitometer (Joko model PAN-II Tokyo).

Protein determination. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard protein.

Preparation of antiserum. Hyperimmune antiserum to the purified NP was made in the rabbit. The NP (0.5 mg) was emulsified with Freund's complete adjuvant and injected intramuscularly. A second intramuscular injection (0.5 mg of the NP in 0.5 ml saline) was given one week later as a booster. Blood samples were taken from the marginal ear vein 3 weeks after the second injection.

Human sera. Paired sera for sero-diagnosis were obtained from children with naturally occurring cases of influenza A infections in early 1976 and early 1978. Five patients were selected for a follow-up study of anti-NP antibody after infection by influenza A virus. All of these patients became ill almost simultaneously with influenza virus infection on December 30th, 1975.

RESULTS

Methods of Isolation and purification of NP antigen

The starting material of the NP antigen was prepared in the allantoic fluid of 10-day-old fertile eggs infected 72 hr previously with the Hong Kong type influenza A virus. The fluid was precentrifuged at 3,000 rpm for 30 min and the virus in the supernatant was concentrated by ultracentrifugation at $186,600 \times g$ for 1 hr. The pellet was suspended in TSB, then ultracentrifuged on a 10% and 50% sucrose cushion. Viruses recovered from the interface between the 10% and 50% sucrose solutions were centrifuged on a sucrose linear gradient (10%–50%). The virus fractions recovered from a white opalescent band midway down the gradient (about 40% sucrose) were diluted approximately 10 times with TSB. After pelleting by ultracentrifugation at $186,000 \times g$ for 1 hr, the virus was resuspended in TSB, solubilized and disrupted with 1.0% Triton X-100 and ultrasonicated at 10 KHz for 1 min. The NP was recovered from the pellet fraction after ultracentrifugation at $186,600 \times g$ for 1 hr. Further purification to remove any residual impurities was performed by preparative electrophoresis. A 100 μ l aliquot of the NP preparation was applied as a line to a 20 cm-wide strip of cellulose acetate membrane (Gelman, Michigan, USA) and electrophoresed at 10 V/cm in barbital buffer, pH 8.5, containing 0.01% SDS. After electrophoresis, the strip was cut into appropriate pieces and the NP protein eluted with distilled water. The purification method is summarized in Fig. 1.

Fig. 1. Purification procedure of NP antigen.

Verification of the purity of the NP preparation

The purity of the NP was examined by both SDS-PAGE and immuno-precipitation. The densitometric pattern of the purified NP is illustrated in Fig. 2. The NP gave a single band in the gel and migrated at a rate corresponding to a polypeptide with a molecular weight of about 60,000. The purity of the NP preparation was indicated to be 95% or more. In attempts to demonstrate the immunological purity of the NP, whole virus antigen, which was solubilized by Triton X-100 and ultrasonic treatment, and anti-NP rabbit serum were prepared (Fig. 3a). Solubilized whole virus antigen gave a single precipitin line against the anti-serum. Furthermore, the anti-serum failed to give precipitin lines with the other three viral components (HA, NA and M). The rabbit anti-serum and human convalescent serum from influenza infections were tested against purified NP

Fig. 2. Densitometric tracing of Coomassie blue stained SDS-polyacrylamide gel of the NP from influenza virus.

antigen. A well-defined single precipitin line was shown between the anti-serum and human serum. This line was continuous (Fig. 3b). These physico-chemical and immunological results showed that the NP preparation had been completely isolated from other viral proteins and it was possible to detect the NP antibody in human serum.

Fig. 3. Immuno double diffusion reaction of influenza antigens with rabbit anti-NP sera and human serum. Diluted antiserum ($\overline{\text{NP}}$) was shown to react with solubilized whole virion (whole) at $\overline{\text{NP}}\times 1$ =original; $\overline{\text{NP}}\times 1/2$ =diluted 1:2; $\overline{\text{NP}}\times 1/4$ =diluted 1:4; $\overline{\text{NP}}\times 1/8$ =diluted 1:8 (3a). Purified NP (NP) reacted with anti-serum and human serum (human), (3b).

Concentrations of NP antigen for IDD and SRD

The purified NP at a concentration of 150 mcg per ml was found to give the best results in IDD reactions with human convalescent sera from individuals with recent influenza A infection. Twenty μg protein per ml mixed with gel were used for the quantitative assay of anti-NP antibody by SRD. The detection of human anti-NP antibody by IDD and SRD was carried out by using each concentration of purified NP antigen.

Application of the IDD and SRD techniques for the detection of anti-NP antibody

Comparison of the sero-diagnosis by anti-HA and anti-NP antibody. In early 1976, 38 paired sera, obtained from children with naturally occurring influenza infections caused by A/Miyagi/1/76 virus (which is an antigenic drift strain from A/Victoria/3/75) were tested by IDD and HAI. In 19 cases, infections were revealed as shown by a twofold or greater rise in HAI against A/Victoria/3/75 strain. Of these cases, anti-NP antibody was detected in all the convalescent sera. In the remaining 19 cases, however, there was no significant rise in HAI titer and in 13 of these cases the anti-NP antibody became positive (shown in Table 1). It is worthy to note that in 32 of the 38 cases anti-NP antibody was detected by

TABLE 1. *Detection rate by IDD and HAI*

Test method	Detection number/total number	Detection rate (%)
HAI	19*/38	50
IDD	32 /38	84

* A two-fold or greater rise in HAI titer with paired sera.

the IDD test. These findings suggest that serodiagnosis by IDD is superior to that by HAI when investigating recent influenza infections caused by antigenic drift strains.

Comparison of infection rates by HA and NP antibody. An unusual outbreaks of a mixed epidemic of A/Texas/1/77 like strain (H3N2) and A/USSR/90/77 like strain (H1N1) occurred throughout Miyagi prefecture in early 1978. Paired sera were obtained from children who were ill with naturally occurring influenza infections caused by either and/or both of A/Texas/1/77 like strain and A/USSR/90/77 like strain. The infection rate by anti-HA antibody to the A/Texas/1/77 strain was about 29% of the total sero-diagnosis and to the A/USSR/90/77 like strain was about 62%. This included a double infection rate of about 18%. The real infection rate by the HAI tests was about 73%. However, sero-diagnosis using the anti-NP antibody as indicated by the SRD method showed an infection rate of 83% (see Table 2). This finding clearly suggests that the detection of the anti-NP antibody has considerable potential value in sero-surveys of epidemics caused by different strains of HA antigen.

TABLE 2. *Serologic diagnosis of anti-NP antibody by SRD and of anti-HA antibody by HAI*

Detection method	Test antigens	Infection number/total number	Infection (%)
HAI	A/Texas/1/77	13*/44	29
	A/USSR/90/77	28*/45	62
SRD	NP	35 /42	82

* A two-fold or greater rise of HAI level between acute and convalescent sera.

Duration of NP antibody after infection

Five patients under clinical observation developed detectable HA and NP antibody levels (Fig. 4). The NP antibody was detected during a period of 12 months or more in case 1, 10 months in case 2, 8 months in case 3, 1 month or less in cases 4 and 5. These results show that antibodies to the NP antigen can be detected for long periods of one year or more by using the IDD technique.

DISCUSSION

A procedure for the isolation of nucleoprotein from influenza A virus in a pure form by solubilization with Triton X-100 followed by SDS-cellulose acetate

fig. 4. A follow-up study of anti-NP antibody and anti-HA antibody in individual cases. ++, +, ±, and - indicate strong, moderate, weak and negative of IDD reaction. In these cases HAI titer (●—●) was indicated between 1:16 and 1:128.

electrophoresis is described. The physicochemical purity of the isolated NP was confirmed by SDS-PAGE and the immunological purity was shown by immunoprecipitation tests. Up to this date, other procedure for the isolation of nucleoprotein from the influenza virion have been reported. In general, there are two points to the procedure: first, the removal of the envelope antigens (hemagglutinin and neuraminidase), and second, the distinction between nucleoprotein and membrane protein. Some researchers use proteolytic enzymes, i.e. protease or negarse and others use detergents, i.e. sarkosyl, deoxy sulphate etc. (Laver 1971; Kendal and Echert 1972; Kendal and Kiley 1973; Stanlay and Crook 1973; Backmayer 1975). The most useful method for the distinction between nucleoprotein and M-protein is believed to be electrophoresis on cellulose acetate membrane or poly-acrylamide. Taking these points into consideration, we chose the detergent (Triton X-100) and cellulose acetate membrane for isolation procedure which seems to be reproducible and quantitative. This immunoreactive NP was also utilized to detect the antibody to the NP in human sera and to determine whether the influenza infections occurred individually. In 1976, when

the minor antigenic drift to A/Miyagi/1/76 from A/Victoria/3/75 occurred, the HAI test using A/Victoria/3/75, which at that time was commonly used as the standard antigen, detected 19 recent infection cases of Hong Kong influenza virus (H3N2) among 38 students. On the other hand, IDD of the purified NP revealed 32 infection cases of influenza. In 1978, when two different influenza A viruses (H3N2 and H1N1) caused the pandemic at the same time (Yamane et al. 1978), the infection ratios observed among students by the HAI test and SRD test to the NP were markedly different. The infection ratios of the A/Texas/1/77 strain and A/USSR/90/77 strain were shown to be 29% and 62%, respectively, by HAI tests. In addition, 19% of the cases were observed to be double infections. Therefore, we can calculate the total infection ratios of A/Texas/1/77 and/or A/USSR/90/77 to be about 72%. However, the SRD test to the NP revealed that 83% of the students were infected with influenza A virus. Therefore, if it is unnecessary to determine the pandemic strain and if it is necessary to determine whether an influenza pandemic is in progress or not, as immediately as possible, these etiological diagnostic procedures for the detection of the anti-NP antibody are useful. Additionally, contrary to our expectations, even several months after infection the IDD test could demonstrate the anti-NP antibody in the sera. Taking these findings into consideration, it has now become possible to carry out sero-epidemiological studies of the mass population using the nucleoprotein.

The principal advantage of the detection of NP antibody by precipitation test can be summarized as follows: 1. It has now become easy to screen infection cases of influenza A virus with no interference from antigenic shift or drift. 2. The results are believed to be unaffected by vaccination, especially of the so-called "split" vaccine used in Japan. 3. It is not necessary to remove non-specific inhibitors which are the most serious problem in the HAI test. 4. From the technical aspects, this procedure is very easy and requires only 5–10 μ l of sera. Also, it is possible to assay many individual sera at the same time.

A collaborative study is in progress in which the immuno double diffusion test and single radial immunodiffusion test will be used for serological and epidemiological studies of influenza infections.

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References

- 1) Backmayer, H. (1975) Selective solubilization of hemagglutinin and neuraminidase from influenza virus. *Intervirology*, **5**, 260–272.
- 2) Davis, B.J. (1964) Disc electrophoresis II. Method and application to human serum protein. *Ann. N.Y. Acad. Sci.*, **121**, 404–427.
- 3) Hirst, G.K. (1941) Agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science*, **94**, 22–23.

- 4) Kendal, A.P. & Echert, E.A. (1972) The preparation and properties of ^{14}C -carboxiamino-methylated subunits from A2/1957 influenza neuraminidase. *Biochem. biophys. Acta (Amst.)*, **258**, 484-495.
 - 5) Kendal, A.P. & Kiley, M.P. (1973) Neuraminidase: Peptide changes associated with antigenic divergence between early and late N2 Neuraminidases. *J. Virol.*, **12**, 1482-1490.
 - 6) Kilbourne, E.D. (1975) A comparison of influenza A, B and C virus. In: *The Influenza Viruses and Influenza*, Academic Press, New York, pp. 5-8.
 - 7) Laver, W.G. (1971) Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. *Virology*, **45**, 275-288.
 - 8) Lief, F.S. & Henle, W. (1959) Methods and procedures for use of complement-fixation technique in type- and strain- specific diagnosis of influenza. *Bull. Wld Hlth Org.*, **20**, 411-420.
 - 9) Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall R.J. (1951) Protein Measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265-275.
 - 10) Palmer, D.F., Dowdle, W.R., Coleman, M.T. & Schild, G.C. (1975) Hemagglutination inhibition test and neuraminidase inhibition test. *Advanced Laboratory Technique of Influenza Diagnosis*, U.S. Department of Health, Education and Welfare, pp. 25-62 and 107-122.
 - 11) Salk, L.E. (1944) Simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. *J. Immunol.*, **49**, 87-98.
 - 12) Schild, G.C. (1972) Evidence for a new type specific structural antigen of influenza virus particles. *J. gen. Virol.*, **15**, 99-103.
 - 13) Schild, G.C. & Pereira, H.G. (1969) Characterization of the ribonucleoprotein and neuraminidase of influenza A viruses by immunodiffusion. *J. gen. Virol.*, **4**, 355-363.
 - 14) Schild, G.C., Oxford, J.S. & Virelizier, J.L. (1975) International symposium on immunity to infections of the respiratory system in man and animals. London. *Develop. Biol. Standard.*, **28**, 253-272.
 - 15) Stanlay, P. & Crook, N.E. (1973) The polypeptide of influenza virus VIII. Large-scale purification of the hemagglutinin. *Virology*, **56**, 640-645.
 - 16) Stuart-Harris, C.H. & Schild, G.C. (1976) The antigens of the influenza viruses. In: *Influenza*, Edward Arnold, Ltd., London, pp. 51-53.
 - 17) Webster, R.G. & Laver, W.G. (1971) Antigenic variations in influenza virus Biology and Chemistry. *Prog. med. Virol.*, **13**, 271-338.
 - 18) Yamane, N., Arikawa, J., Odagiri, T., Sueno, N. & Ishida, N. (1978) Isolation of three different influenza A viruses from an individual after probable double infection by H3N2 and H1N1 viruses. *Jap. J. med. Sci. Biol.*, **31**, 431-434.
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